Apoptosis, Fas and systemic autoimmunity: the MRL-*lpr/lpr* model

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Introduction

The death of lymphocytes by apoptosis is an important mechanism for maintaining self-tolerance and homeostasis in the immune system. Failure of apoptotic cell death has long been postulated as a cause of loss of self-tolerance and the development of autoimmunity. Mice expressing the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) mutations develop an accelerated, lupus-like systemic autoimmune disease. The findings that these single-gene defects are due to abnormalities in the cell death associated gene, *fas*, or in the Fas ligand (FasL), have formally established the importance of apoptotic cell death in preventing autoimmunity. These two animal models represent the first autoimmune diseases in which the major underlying genetic abnormality is known. In this paper, we review the role of apoptosis in the maintenance of self-tolerance, the genetic and immunologic basis of disease in MRL-*lpr/lpr* mice, and the relevance of this model in other animal and human models of systemic autoimmunity.

Lymphocyte apoptosis and tolerance to self antigens

Apoptotic cell death is characterized by fragmentation of DNA into nucleosome-sized fragments, nuclear dissolution and cell shrinkage. This process occurs in lymphocytes under several conditions, some of which appear to be genetically programmed (and hence are correctly cited as examples of 'programmed cell death'), and others are due to withdrawal of growth factors or as a consequence of antigen receptor-mediated activation [1]. During the development of T lymphocytes in the thymus, cells that express receptors that fail to recognize self MHC-encoded molecules, and are therefore not positively selected, undergo apoptotic programmed cell death. Immature T cells that express high-affinity receptors for self peptide + self MHC are believed to undergo activation-induced apoptosis as a result of antigen recognition. This is the principal mechanism of negative selection (clonal deletion) of self antigen-reactive thymocytes [2–4]. It can be mimicked by exposing immature T cells to antibodies that crosslink T cell receptors [5,6]. Similar mechanisms are believed to regulate the development of the mature, self-tolerant B-cell repertoire, although this process is not as well understood as it is for T lymphocytes [7].

After lymphocytes mature and leave the generative tissues (thymus and bone marrow), they are functionally competent, i.e. capable of responding to antigenic stimulation by proliferating and differentiating into effector cells. Among the progeny of antigen-stimulated lymphocytes, only a small fraction develop into functional effector cells and memory cells, and the majority probably die by apoptosis [8]. This is a process of activation-induced cell death that may be enhanced by the exposure of antigen-stimulated lymphocytes to growth factors, such as interleukin (IL)-2 in the case of T cells [9]. In mice, administration of large doses of anti-T cell receptor antibodies or superantigens, such as staphylococcal enterotoxins, results in the deletion of T cells that express antigen receptors which specifically bind these antibodies or superantigens [10–12]. This is probably also due to

Abbreviations

CTL—cytotoxic T lymphocyte; FasL—Fas ligand; *gld*—generalized lymphoproliferative disease; IL—interleukin; *lpr*—lymphoproliferative gene; SLE—systemic lupus erythematosus; TNF—tumor necrosis factor.

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activation-induced apoptosis, and may be an exaggerated version of the phenomenon that normally occurs in clones of antigen-specific lymphocytes that encounter the antigen. Activation-induced cell death in mature lymphocytes is a homeostatic mechanism that functions to regulate the size of antigen-stimulated clones. It is not known whether the same mechanism is responsible for the maintenance of peripheral tolerance to tissue-specific self antigens, although recent findings in MRL-*lpr/lpr* mice indicate that apoptotic cell death may indeed be an important mechanism of peripheral self-tolerance.

Genetic control of apoptosis in lymphocytes

A large number of genes that determine apoptotic programmed cell death have been identified in *Caenorhabditis elegans* [13], and more recently in *Drosophila* sp. [14]. Much of the interest in these genes has focused on their role in embryogenesis and the development of specialized tissues, including the central nervous system. Although homologous genes are being identified in mammals, it appears that apoptosis in lymphocytes and other hemopoietic cells is regulated by unique genes. These include members of the tumor necrosis factor (TNF) receptor/*fas* superfamily, proto-oncogenes such as *bcl-2* and *c-myc*, and genes that regulate the cell cycle, such as *p53* [15].

The TNF receptor/*fas* superfamily

The Fas protein was first identified as a membrane protein and as a target for antibodies that induced apoptosis in cell lines [16]. The human protein called APO-1, which was also identified as a target for an antibody that induced apoptosis, is identical to Fas [17], as is CD95. Fas is a member of a family of proteins with 12 known members, including the p55 and p75 components of the TNF receptor, CD40, OX40, CD27 and CD30 [18••]. All members of the family are type I membrane proteins containing homologous extracellular domains with three to six cysteine-rich 40 amino acid long pseudorepeats, and cytoplasmic domains of variable length without significant sequence homology (Fig. 1). Most of these proteins can be produced in soluble forms, which are usually generated by proteolysis of the membrane proteins. The ligands for these proteins are all type II membrane molecules, with extracellular carboxy-terminal regions and intracellular amino termini (Fig. 1). Eight of these ligands have been cloned; sequence homologies among them are limited to the carboxyl terminal ~150 residues, which presumably form the receptor-binding regions. All the ligands can also be expressed in membrane-associated or secreted forms.

The *fas* gene encodes a ~45 kDa protein. It is expressed in activated mature T cells, thymus, liver, ovary, lung and heart [19]. FasL is a ~40 kDa protein; FasL RNA is present in testis, small intestines, kidney, lung, activated splenocytes, and, to a lesser degree, activated thymocytes, but there is little information available about protein expression [20••,21].

Members of the TNF receptor/*fas* superfamily all regulate the growth, differentiation and apoptotic cell death of lymphocytes. Binding of FasL or anti-Fas antibodies to cell-surface Fas induces apoptosis, much like the binding of TNF to its receptor on target cells. The process of Fas-mediated cell death is essential for maintaining T-cell tolerance to self antigens, as revealed by analysis of the MRL-*lpr/lpr* mouse strain. In addition, apoptosis induced by cytolytic T lymphocytes (CTLs) is also mediated by FasL produced by the CTLs binding to Fas on target cells. This mechanism of target-cell killing complements osmotic lysis induced by the insertion of perforin into target cell membranes [22•]. In fact, the principal mechanism of cytolysis by $CD4^+$ CTLs is Fas-mediated [23•]. In contrast to the FasL–Fas interaction, CD40L or anti-CD40 antibodies protect CD40-expressing B cells from death, and stimulate the growth and differentiation of these cells [24–26]. In some cells, TNF-α also induces proliferation and not death [27], and even Fas engagement has been shown to increase the proliferation of some T lymphocytes [28]. The mechanisms by which different TNF receptor/*fas* superfamily members transduce ligand-induced signals and the biochemical basis for the distinct functional consequences of ligation of these receptors are entirely unknown. The regulation of Fas and FasL expression in lymphocytes is also not yet fully defined, and this is an area of intensive investigation at present. Activated T helper (Th) 1 clones may express higher levels of FasL than most Th2 clones [29], but it is unlikely that FasL expression is restricted to any one T cell subset. How cytokines and costimulators influence Fas and FasL expression and function is another, largely unresolved issue.

Other genes that regulate apoptosis in lymphocytes

The proto-oncogene, *bcl-2*, promotes cell division largely by inhibiting programmed cell death. Transgenic mice that overexpress *bcl-2* in lymphoid cells show prolonged antibody responses to immunization, and enhanced generation of memory B cells [30,31]. Targeted disruption of the *bcl-2* gene results in a dramatic postnatal involution of both thymus and spleen, secondary to widespread apoptotic cell death [32]. Fas-mediated apoptosis may be counteracted by overexpression of *bcl-2* in cell lines, but the mechanism of this effect is not known [33]. The proto-oncogene *c-myc* generally inhibits apoptosis in cell lines. The tumor suppressor p53 is required for apoptosis induced in thymocytes by ionizing radiation [34]. Recently, Nur 77, an orphan steroid receptor, has been shown to be required for activation-induced apoptosis in immature T cells [35,36]. Many of these apoptosis-regulating genes were first identified in tumors, and postulated to play critical roles in the prolonged survival and uncontrolled growth of neoplastic cells. Subsequent studies have implicated these same genes in the growth and survival of normal cells, but the importance of *bcl-*

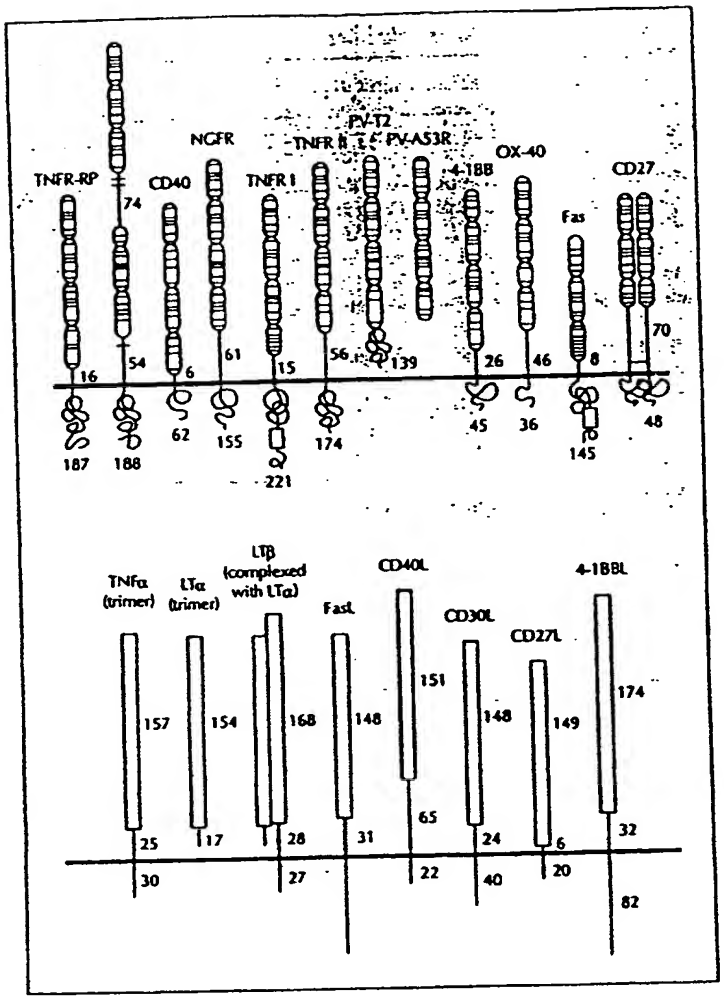

Fig. 1. Members of the TNF receptor/Fas superfamily (top) and the TNF/FasL superfamily (bottom). Published with permission [18••].

2, *c-myc*, *p53* and *Nur 77* in the physiologic regulation of immune responses and self-tolerance is not yet established.

Autoimmunity caused by the *lpr* and *gld* mutations

The spontaneous lupus-like autoimmune disease of MRL-*lpr/lpr* and *gld* mice has provided a powerful model for analyzing the mechanisms of self-tolerance and autoimmunity. Although considerable confusion has arisen about the basis of autoimmunity in these strains, recent molecular and immunologic analyses have provided important insights.

Immunologic abnormalities in MRL-*lpr/lpr* and *gld* disease

MRL mice homozygous for the *lpr* gene (MRL-*lpr/lpr*) develop multiple autoantibodies, including antinucleoprotein antibodies, rheumatoid factor, and immune complex nephritis, which is fatal by ~6 months [37]. In addition, after ~6 weeks of age, the mice develop progressive lymphadenopathy due to the accumulation of an unusual population of $CD4^-$ $CD8^-$ $TCR\alpha\beta^+$ $CD3^+$ (double-negative) T cells that also express the CD45R isoform called B220, normally a marker of the B-lymphocyte lineage. These double-negative T cells do not proliferate and are inert to extrinsic stimuli, including anti-CD3 antibodies and IL-2. One recent paper shows that the double-negative cells can be induced to proliferate when cultured with anti-CD3 and an activating antibody against CD28, TCR for co-stimulators [38], but

the significance of this observation is not known. Since the cells that cause lymphadenopathy are not actively proliferating, the term 'lymphoproliferation' is actually a misnomer. MRL-*lpr/lpr* mice also contain an abnormal number of autoreactive CD4+ T cells, which are detectable before the development of lymphadenopathy [39]. CD4+ T cell lines from MRL-*lpr/lpr* mice are resistant to cell death induced by high concentrations of anti-CD3 or anti-TCR antibodies [40•,41•,42•], and these T cells have a striking growth advantage over normal CD4+ T cells in mixed cultures stimulated by alloantigens or other T-cell activators [43]. Autoreactive CD4+ T cells are capable of helping syngeneic B lymphocytes by inducing proliferation and antibody secretion, whereas the double-negative T cells are not functional in these assays (GG Singer, A Marshak-Rothstein, AK Abbas, unpublished data). It is not yet known whether particular cytokines play obligatory roles in the helper function of autoreactive CD4+ cells.

Mice homozygous for the *gld* gene develop a phenotypically very similar disease, characterized by autoantibody production, immune complex nephritis, lymphadenopathy due to the accumulation of double-negative T cells, and a high frequency of autoreactive CD4+ T lymphocytes [37,43]. The responses of *gld* T cells to extrinsic stimuli have not been analyzed in detail.

Several lines of evidence indicate that autoimmunity in MRL*lpr/lpr* mice is dependent on CD4+ helper T cells, and can be segregated from the lymphadenopathy. Treatment of MRL-*lpr/lpr* mice with an antibody against CD4 prevents or retards disease [44]. In contrast, anti-CD8 antibody treatment prevents lymphadenopathy but not autoimmunity [45]. More definitive proof has come from breeding studies. MRL-*lpr/lpr* mice bred with mice deficient in class II MHC do not develop autoimmunity, probably because class $II^{-/-}$ animals lack CD4+ T cells, but again lymphadenopathy is not affected [46•]. The same result is seen in $CD4^{-/-}$ MRL-*lpr/lpr* mice, whereas $CD8^{-/-}$ MRL-*lpr/lpr* mice do develop autoantibodies and show variable degrees of lymphadenopathy (D Roh and TW Mak, personal communication). Thus, the conclusion of these analyses is that autoantibody production is dependent on autoreactive CD4+ helper T cells, whereas lymphadenopathy may be due to abnormalities in either CD4+ or CD8+ T cells (and is not, therefore, abolished in any of the mice described above). It is likely that the *lpr* mutation affects cell lineages other than CD4+ T lymphocytes. For instance, in chimeras between *lpr/lpr* and normal strains, B cells derived from the former have a striking growth advantage and are required for the development of autoimmunity, suggesting that *lpr/lpr* B cells are also intrinsically abnormal [47,48].

If CD4+ autoreactive T cells are critical for the development of autoimmunity, the key question that arises is why do MRL-*lpr/lpr* and *gld* mice contain large numbers of autoreactive Th lymphocytes?

Genetic basis of autoimmunity in MRL-*lpr/lpr* and *gld/gld* mice

The single most important advance in our understanding of autoimmunity in these strains has been the identification by Nagata's laboratory of the *lpr* mutation as a defect in the *fas* gene [49]. This abnormal *fas* gene contains a retrotransposon insertion in the second intron, leading to aberrant splicing and premature termination of transcription [50•,51•]. As a result, cells from MRL-*lpr/lpr* mice produce greatly reduced or undetectable *fas* transcripts. Although less is known about protein expression, the available data indicate that Fas protein is also essentially undetectable in *lpr/lpr* mice. Formal proof that the *fas* defect causes both autoimmunity and lymphadenopathy has come from the demonstration that the disease of MRL-*lpr/lpr* mice can be cured if a normal *fas* gene is expressed as a transgene in the T cells of these animals [52••].

The cloning of the gene encoding the FasL [20••,21] was rapidly followed by the finding that *gld* is a point mutation in the extracellular domain of the gene encoding FasL [53••]. The identity of the *gld* gene has been confirmed by positional cloning [54•]. Moreover, it has been shown that this abnormal FasL is incapable of inducing apoptosis in Fas-expressing target cells. The demonstration that *lpr* and *gld* are abnormalities in the genes encoding a receptor–ligand pair provides the structural explanation for the long-held view that these autoimmune strains are caused by abnormalities in a complementary ligand : receptor pair [55].

However, the disease of MRL-*lpr/lpr* mice, although clearly *fas*-mediated, is significantly modified by background genes. For instance, the levels of serum autoantibodies and the severity of nephritis vary depending on the background strain [37]. Furthermore, the MRL strain is itself autoimmune-prone, and the *lpr* defect markedly accelerates the development of autoimmunity.

Mechanisms of autoimmunity in MRL-*lpr/lpr* mice

On the basis of the known immunologic and genetic abnormalities in this strain, it is reasonable to postulate that a failure of apoptotic cell death in CD4+ T lymphocytes is responsible for the persistence of pathogenic autoreactive Th cells and the subsequent production of autoantibodies. Autoreactive T cells may persist either because of an abnormality in negative selection in the thymus or because of a peripheral defect. Recent data indicate that thymic selection, assessed by the expression of TCR Vβs, occurs normally in the thymus of MRL-*lpr/lpr* mice [56•]. More direct evidence that the defect is peripheral and not thymic has come from *lpr/lpr* mice expressing a transgene-derived TCR specific for a known peptide + class II MHC. TCR-expressing CD4+ T cells develop normally in these mice but are resistant

to activation-induced apoptotic cell death. More importantly, the systemic administration of high doses of peptide results in comparable deletion of intrathymic T cells expressing the transgenic TCR in *lpr/lpr* and normal (+/+) mice, whereas mature T cells in peripheral lymphoid tissues are deleted in +/+ but not in *lpr/lpr* animals [57•]. These results indicate that Fas plays an essential role in activation-induced death of mature T cells in the periphery, but not in negative selection in the thymus. Therefore, one can postulate that normally, some self antigen-reactive T cells may mature and enter peripheral tissues, where an encounter with self antigens leads to FasL/Fas-mediated apoptosis. In MRL-*lpr/lpr* mice, failure of apoptosis results in the persistence of these autoreactive T cells, which help autoreactive B cells that are not deleted. The B cells are thus stimulated to produce high-affinity autoantibodies (Fig. 2). The insensitivity of *lpr/lpr* B cells to Fas/Fas L-mediated cytotoxicity may also contribute to autoantibody production [58]. Promoting activation-induced cell death may restore peripheral tolerance even in Fas- or FasL-deficient mice. This is the likely mechanism by which infection of MRL-*lpr/lpr* mice containing IL-2, but not IL-4 or granulocyte-macrophage colony-stimulating factor, viral vectors cures autoimmunity [59,60], since IL-2 is known to program T cells for apoptosis [9]. Failure of peripheral activation-induced T cell death is also the probable explanation for the relative inefficiency of superantigens to delete specific T cells in MRL-*lpr/lpr* mice [61].

Although the model presented in Fig. 2 may be the simplest explanation for the development of autoimmunity in *lpr/lpr* (and, by inference, *gld/gld*) strains, many issues remain unresolved. First, the relationship between persistent autoreactive T cells and the accumulation of double-negative cells in lymphoid organs is not known. One possibility is that chronic stimulation of autoreactive T cells by self antigens leads to compensatory downmodulation of CD4 or CD8 co-receptors and accumulation of inert, 'double-negative' cells. Since this could happen in either $CD4^+$ or $CD8^+$ populations, selectively depleting either subset may not prevent lymphadenopathy. Alternatively, the double-negative T cells may arise from some abnormal thymic population that emigrates to the periphery [62]. The role of other cell surface molecules, such as CD2, in the accumulation of functionally anergic double-negative cells and in apoptosis in B cells has also been suggested [63,64], but its relevance to autoimmunity is unknown. Second, it is not clear why autoreactive T cells persist in an inappropriate way in *lpr/lpr* mice, but T-cell responses to immunization with foreign antigens in adjuvants do not show abnormally prolonged kinetics and are not aberrantly high (GG Singer, AK Abbas, unpublished data). Identifying the self antigens that elicit autoimmune reactions, and the way these antigens are presented to T cells, may provide important clues about the activation of autoreactive cells. Finally, the contributions of other components of the immune system, e.g. B cells, or of background genes, are not understood.

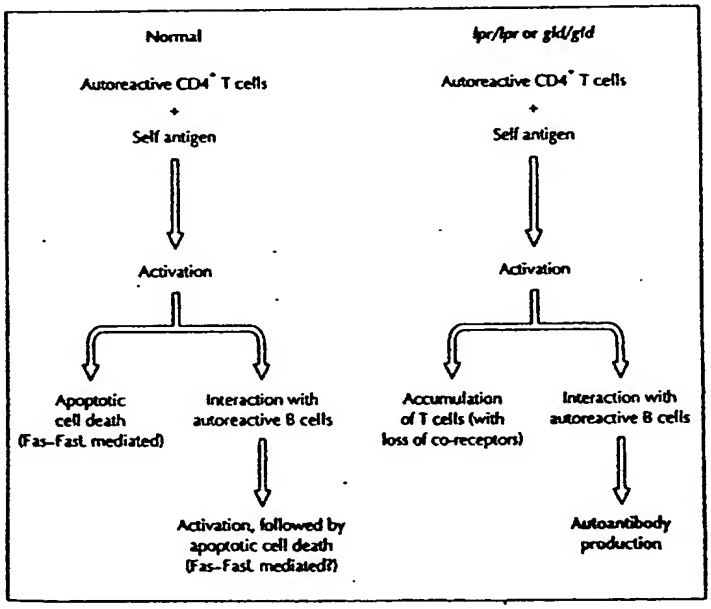

Fig. 2. A hypothetical model for the development of autoimmunity and lymphadenopathy as a result of the *lpr* and *gld* mutations. The postulated normal sequence of lymphocyte activation and regulation is shown for comparison.

Lymphocyte apoptosis and other models of autoimmunity

The relevance of the MRL-*lpr/lpr* model to human lupus and to other autoimmune disorders is an issue of great interest at present. Human systemic lupus erythematosus (SLE) is not caused by a single recessive gene, and is usually not associated with lymphadenopathy. By these criteria, SLE is not the same disease as the autoimmunity of MRL-*lpr/lpr* mice. Nevertheless, Mountz's group has recently found that some SLE patients have elevated serum levels of soluble Fas, which can block Fas-mediated apoptosis [65••]. This raises the possibility that secretion of soluble Fas may be the basis of autoimmunity in SLE, although the alternative, that high serum concentrations of soluble Fas are a result of lymphocyte activation, has not been excluded. Undoubtedly, many similar studies of SLE patients will be reported in the near future.

Regulating apoptotic cell death may be a general mechanism for inducing or ameliorating autoimmunity [15]. One line of transgenic mice constitutively expressing *bcl-2* in B lymphocytes develops an autoimmune syndrome very similar to that of MRL*lpr/lpr* mice [30]. The likely mechanism is that overexpression of *bcl-2* prevents apoptotic cell death in self-reactive B lymphocytes. Conversely, repeated administration of a self antigen, myelin basic protein, reduces the autoimmune reactions specific to this protein, probably by activating T cells to produce large amounts of IL-2, and thus promoting apoptotic cell death [65••]. This raises the possibility that strategies for inducing apoptosis in lymphocytes may be of therapeutic benefit even in diseases associated with defects in the Fas–FasL pathway.

Conclusions

In summary, although MRL-*lpr/lpr* and *gld/gld* mice develop systemic disease due to multiple autoantibodies and immune complexes, they do not develop organ-specific, T cell mediated, autoimmune disorders. This suggests that different mechanisms may be responsible for maintaining tolerance to disseminated and tissue-specific self antigens. For instance, T-cell tolerance to widely disseminated self antigens may be due to negative selection in the thymus or Fas-dependent cell death in peripheral sites. Failure of the latter is the cause of the systemic disease of *lpr/lpr* and *gld/gld* mice. In contrast, tissue-restricted self antigens may normally be presented by co-stimulator deficient, antigen-presenting cells, and may thus induce anergy in potentially autoreactive T-cell clones that have attained maturity. This process of clonal anergy does not involve Fas–FasL interactions. Elucidation of the mechanisms of self-tolerance to diverse autoantigens will provide a rational basis for devising therapeutic strategies for spontaneous autoimmune diseases.

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